Estradiol Partially Recapitulates Murine Pituitary Cell Cycle Response to Pregnancy

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Because pregnancy and estrogens both induce pituitary lactotroph hyperplasia, we assessed the expression of pituitary cell cycle regulators in two models of murine pituitary hyperplasia. Female mice were assessed during nonpregnancy, pregnancy, day of delivery, and postpartum. We also implanted estradiol (E2) pellets in female mice and studied them for 2.5 months. Pituitary weight in female mice increased 2-fold after E2 administration and 1.4-fold at day of delivery, compared with placebo-treated or nonpregnant females. Pituitary proliferation, as assessed by proliferating cell nuclear antigen and/or Ki-67 staining, increased dramatically during both mid-late pregnancy and E2 administration, and lactotroph hyperplasia was also observed. Pregnancy induced pituitary cell cycle proliferative and inhibitory responses at the G1/S checkpoint. Differential cell cycle regulator expression included cyclin-dependent kinase inhibitors, p21Cip1, p27Kip1, and cyclin D1. Pituitary cell cycle responses to E2 administration partially recapitulated those effects observed at mid-late pregnancy, coincident with elevated circulating mouse E2, including increased expression of proliferating cell nuclear antigen, Ki-67, p15INK4b, and p21Cip1. Nuclear localization of pituitary p21Cip1 was demonstrated at mid-late pregnancy but not during E2 administration, suggesting a cell cycle inhibitory role for p21Cip1 in pregnancy, yet a possible proproliferative role during E2 administration. Most observed cell cycle protein alterations were reversed postpartum. Murine pituitary meets the demand for prolactin during lactation associated with induction of both cell proliferative and inhibitory pathways, mediated, at least partially, by estradiol. (Endocrinology 153: 5011–5022, 2012)

Robust polyclonal lactotroph proliferation is induced during pregnancy (1–5), and in rats pituitary proliferation peaks at delivery and returns to prepartum levels several days after lactation initiation (1–3). After lactation, partial or complete regression of redundant lactotrophs occurs (6, 7). Pharmacological estradiol (E2) treatment also induces early lactotrophic hyperplastic responses, angiogenesis, and prolactinoma development in rats, coincident with pituitary tumor-transforming gene (PTTG), basic fibroblast growth factor, and vascular endothelial growth factor induction (8, 9). Because E2 also potentiates lactotroph proliferation and cell cycle modulation during pregnancy, we studied effects of pregnancy and E2 administration in murine pituitary glands.

Cyclin-dependent kinases (CDK), critical regulators of cell cycle progression, are modulated by fluctuations in activators (cyclins) or inhibitors (CDK inhibitors) (10). Little is known regarding endocrine-mediated molecular mechanisms underlying pituitary cell cycle events during gestation and E2 administration, in part due to several technical limitations. Pituitary glands comprise heterogeneous populations of lactotroph, somatotroph, gonadotroph, corticotroph, thyrotroph, and folliculostellate cell components (11). Accordingly, studies using whole pituitary glands may not reflect events occurring in a single cell subtype. This would be important because cell cycle protein responses to growth factors likely occur predominantly in a subset of specific cells during the G1 and S

Abbreviations: CDK, Cyclin-dependent kinase; E2, estradiol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCNA, proliferating cell nuclear antigen; WT, wild type.
phases. Because only whole pituitary glands can be studied in vivo, single-cell type alterations in these proteins could be masked or diluted by a larger compartment of predominantly noncycling pituitary cells. Finally, no mouse or human lactotroph cell line is available for in vitro study. Insights into mechanisms involved in lactotroph cycle regulation have been obtained from experiments in CDK4-deficient mice, which exhibit decreased lactotroph mass, function, and postnatal proliferation (12, 13). Because CDK4 is an essential regulator of the G₁/S checkpoint, lactotroph cell cycle progression is likely dependent on components that converge on this enzyme.

Hence, it was hypothesized that gestation or E₂ administration would induce pituitary cell cycle progression at the G₁/S checkpoint and an inhibitory cell cycle response only after delivery. This study elucidates a parallel proliferative and inhibitory cell cycle response in both models, possibly restricting pituitary hyperplastic growth. Because CDK inhibitors are shown to be involved in either progression or arrest of the cell cycle at the G₁/S checkpoint, this appears to be a particularly important site of pituitary proliferative control during gestation or E₂ administration. Most of the observed cell cycle alterations during pregnancy were reversed at lactational and postweaning stages. These results elucidate pituitary cell cycle modulation in parallel to pituitary remodeling occurring during pregnancy or E₂ administration.

Materials and Methods

Animals

Wild-type (WT) mice of a C57/BL6 genetic background were maintained in a light- (12 h light, 12 h darkness cycle) and temperature-controlled room. Animals were euthanized using CO₂ chambers, and blood withdrawn directly from the heart, and pituitary glands harvested for analysis. Sera were stored at −80 C until hormone assay. Experiments were approved by the Institutional Animal Care and Use Committee.

Reproductive study

WT virgin females at approximately 8–10 wk of age were impregnated by caging with a WT male. The presence of a vaginal mucus plug was considered as d 0.5 of pregnancy. Males were removed 2 wk later, before parturition.

Female mice were assigned to six reproductive stages: 1) nonpregnant; 2) 2 wk pregnancy [this stage was determined by counting 14.5 (range 13.5–15.5) d after observing a vaginal plug and by embryo morphology consistent with this gestational age]; 3) day of delivery was determined as the 24-h period after pups were delivered, on average at 20.5 (range 19.5–21.5) d of pregnancy; 4) 3 wk lactation (the day of parturition was counted as d 0 of lactation, and pups were weaned on d 21 of lactation and maternal pituitary glands harvested on the same day); 5) 3 wk after weaning (this stage included mice whose pups were weaned 3 wk earlier, and females in groups 4 and 5 with litters smaller than three pups were not included for study); and 6) 3 wk postpartum. In this group pups were euthanized on the day of parturition. Maternal pituitary glands were harvested 3 wk after delivery.

Nonpregnant females were euthanized at approximately 8–10 wk. In other groups, females were euthanized according to their respective reproductive stages. For the weight analysis, the pituitary glands were fixed in 10% formalin and weighed 24 h after collection.

Pellet administration

Eight- to 10-wk-old WT female mice were surgically implanted under isoflurane anesthesia with 17β-estradiol pellet for 90 d (1.5 mg/pellet; Innovative Research of America, Sarasota, FL) or placebo pellet. Mice were euthanized after 70–75 d. Fresh pituitary glands were collected for assays and weighed immediately after collection.

Protein analysis

Each tissue lysate was prepared from four pituitary glands in radioimmunoprecipitation assay buffer (Sigma, St. Louis, MO) containing protease inhibitor cocktail (Sigma). Protein concentrations were measured by bichinchoninic acid protein assay (Thermo Scientific, Swedesboro, NJ). Equal amounts (30 μg) of proteins were separated by NuPage Novex Bis-Tris gels (Invitrogen, Carlsbad CA) and electroblotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were incubated with anti-p15INK4b (1:200; ab53034; Abcam, Cambridge, MA); p16INK4a (1:200; sc-1207; Santa Cruz Biotechnology, Santa Cruz, CA); p18INK4c (1:500; 39-3400; Invitrogen); p19INK4d (1:100; 39-3100; Invitrogen); p21Cip1 (1:200; BD-556431; BD PharMingen, San Diego, CA); p27Kip1 (1:200; BD-556431; BD PharMingen, San Diego, CA); p53 (1:300; ab31333; Abcam); Phos-Rb (1:200; sc-365; Santa Cruz Biotechnology); cyclin A (1:200; sc-396; Santa Cruz Biotechnology); cyclin B1 (1: 500; ab72-100; Abcam); cyclin B2 (1:200; sc-28303; Santa Cruz Biotechnology); cyclin D1 (1:200; ab16663; Abcam); cyclin D3 (1:200; sc-182; Santa Cruz Biotechnology); cyclin E (1:200; sc-481; Santa Cruz Biotechnology); glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1,000; sc-25778; Santa Cruz Biotechnology); and actin (1:20,000; MAB1501; Chemicon, Temecula, CA) antibodies overnight, followed by the corresponding secondary antibodies (1:1,000; GE Healthcare, Indianapolis, IN). Proteins were visualized using the enhanced chemiluminescence Western blot detection reagents (GE Healthcare) super signal Western system (Pierce, Rockford, IL). Scanning densitometry of protein bands was determined by pixel intensity using NIH Image J software (National Institutes of Health, Bethesda, MD) and normalized against that of actin or GAPDH.

Immunofluorescent and reticulin stainings

Pituitary glands were dissected and fixed in 10% formalin for paraffin sectioning. For immunofluorescence analysis, 5-μm paraffin pituitary sections were deparaffinized and rehydrated. Antigen retrieval was performed in 10 mM sodium citrate and 0.25% Tween 20 by incubating slides at 98 C for 40 min and
cooling for 20 min. For nuclear antigens, slides were permeabilized with 1% Triton X-100 in PBS for 30 min. Sections were incubated with blocking buffer for 1 h and then with anti-PCNA (1:50; sc-56; Santa Cruz Biotechnology); Ki-67 (1:1000; ab15580; Abcam); prolactin (1:500; sc-7805; Santa Cruz Biotechnology); GH (1:200; the National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA, and Dr. A. F. Parlow, Harbor-UCLA Medical Center, Los Angeles, CA); and p21 (1:30; BD-536341; BD PharMingen) antibodies at 4°C overnight. Sections counterstained with 4',6'-diamidino-2-phenylindole (300 nM; D3571; Invitrogen). The samples were imaged with Leica TCS/SP spectral confocal scanner (Leica Microsystems, Mannheim, Germany).

PCNA proliferative index was determined using ImageJ software (Image Processing and Analysis in Java; National Institutes of Health) and based on the number of positively stained nuclei divided by the total number of nuclei counted. Three fields each containing approximately 500 cells were counted from each animal and two to three animals from each group analyzed. PCNA-immunoreactive cells were similarly examined to determine the percentage of pituitary cells immunoreactive for both prolactin and PCNA of the total PCNA-immunoreactive cells counted. Silver staining for reticulin fiber detection was performed selectively.

RNA isolation and PCR

RNA samples were prepared from four pituitary glands. Total RNA was isolated using the RNeasy minikit (QIAGEN, Valencia, CA) and eluted in water. cDNA was synthesized from 500 ng of total RNA using 1 μg of oligo(deoxythymidine) primer, SuperScript II reverse transcriptase, and accompanying reagents (Invitrogen). Real-time PCR was amplified in 25-μl reaction mixtures [100 ng template, 0.5 μM of each primer, 10 μM 2× SYBR Green master mix (Applied Biosystems, Foster City, CA)] using 95°C for 1 min, followed by 40 cycles of 95°C for 20 sec and 60°C for 40 sec. GAPDH and 18s RNA were assessed as internal controls.

Radioimmunoassay

Serum concentrations of E2 (picograms per milliliter) were determined using the DSL-4800 assay kit (Diagnostic Systems Laboratories, Webster, TX).

Statistical analysis

Differences between groups were analyzed by *t* test. Values were considered significant when *P* < 0.05.

Results

Pituitary mass and lactotroph proliferation during pregnancy and E2 administration

To address pituitary expansion in the mouse during E2 administration and pregnancy, we first assessed pituitary weights and expression of two proliferation markers, PCNA and Ki-67. Female mouse pituitary mass increased 2- or 1.4-fold with E2 administration or at day of delivery, compared with placebo-treated or nonpregnant females, respectively (*P* < 0.0001 and < 0.001, Fig. 1, A and E). Pituitary to body weight ratio at day of delivery increased by 60% compared with respective weights at nonpregnant stages (0.16 *vs.* 0.10, respectively, *P* < 0.0001, Fig. 1F). This expansion did not reverse after the day of delivery. However, rate of pituitary weight increase stabilized between day of delivery and 3 wk after weaning (pituitary to body weight ratio of 0.16 *vs.* 0.18, respectively, Fig. 1F). At 3 wk postpartum, 3 wk after pup removal at day of delivery, pituitary weight only partially reverted to pregestational levels (*P* < 0.01; see Fig. 2, A and B).

Increased pituitary weight correlated strongly with increased PCNA and Ki-67 immunostaining (Fig. 3). On average, in nonpregnant females 0.8 ± 0.6% of pituitary cells were PCNA positive (Table 1 and Fig. 3E). Conversely, PCNA labeling increased to 4.4 ± 1.6% of cells at day of delivery (*P* < 0.001, Table 1 and Fig. 3F). Ki-67 antigen detection also confirmed increased proliferation during E2 administration and at 2 wk of pregnancy and day of delivery stages *vs.* placebo administration and nonpregnancy, respectively (Fig. 3, P, L, and M vs. Fig. 3, O and K). By Western analysis, increased pituitary PCNA expression and Rb phosphorylation were observed during E2 administration and at 2 wk pregnancy and day of delivery, compared with placebo-treated and nonpregnant females, respectively (Fig. 3, A and B). Furthermore, elevated PCNA and Ki-67 labeling in E2-treated and 2 wk pregnant mice closely mirrored the 2- to 8-fold increases in PCNA and Ki-67 mRNA expression (Fig. 3, C and D). Parallel staining for prolactin and PCNA or Ki-67 showed that lactotroph cells comprised the main proliferating cell type either at 2 wk pregnancy and day of delivery or during E2 administration (Fig. 3, L, M, and P, and Table 1). Prolactin coexpression in PCNA-positive cells increased from 76 ± 25 to 93 ± 6% during nonpregnant and day of delivery stages, respectively (*P* = NS, Table 1). However, the proportion of somatotrophs in the PCNA-positive cells did not change during either nonpregnant or day of delivery stages (*P* = NS, 12 ± 18 and 11 ± 3%, respectively, Table 1 and Fig. 3, I and J).

The results confirm that marked lactotroph proliferation occurred concurrent with increased pituitary weight during mid-late pregnancy, and lactotroph stabilization occurred after the late phase of lactation. During 2.5 months of E2 administration, pituitary replication was evident by increased protein levels, mRNA induction, and immunostaining of proliferation markers. Pituitary proliferation rates during E2 administration also correlated with serum E2 levels (Fig. 1D).
Pituitary expansion during estradiol administration and pregnancy is related to hyperplasia

Anterior pituitary cells were diffusely distributed at 2 wk pregnancy, day of delivery, and 3 wk lactation, and the reticulin fiber network was intact, consistent with the definition of lactotroph hyperplasia (Fig. 4, A–D). A similar pattern was detected in specimens derived from E2- vs. placebo-administered mice (Fig. 4, G vs. F). However, the histological distribution became more nodular at 3 wk lactation (Fig. 4D). Correspondingly, reticulin fiber dissolution accompanied hyperplasia in several fields, but distinct pituitary tumors were not observed. Reticulin fiber distribution at 3 wk postpartum (Fig. 4E) demonstrated a reversal to the nonpregnant pattern (Fig. 4A).

Differentially expressed pituitary cell cycle regulation genes at mid-late gestation

We isolated protein and total RNA samples from female pituitaries at different reproductive stages, including nonpregnant, 2 wk pregnancy, day of delivery, 3 wk lactation, and 3 wk after weaning. We identified several gene products differentially expressed in pituitary lysates compared with nonpregnant controls. The most marked changes were observed at 2 wk of pregnancy. At this stage, elevated protein levels of CDK inhibitors p15INK4b and p21Cip1 were detected (Fig. 5, B and E). In contrast, p16INK4a and p27Kip1 levels were decreased, whereas p18INK4c and p57Kip2 levels were unchanged.
Quantitative PCR confirmation of selected differentially expressed genes concurred with Western blotting results. Specifically, p15INK4b and p21Cip1 increased 2.3- and 5.9-fold, respectively, at 2 wk pregnancy (Fig. 6B), and immunostaining demonstrated increased pituitary cell p21Cip1 expression at 2 wk pregnancy, concurrent with increased Ki-67 labeling (Fig. 6D). Nuclear Ki-67 labeling is reflective of proliferating cells, and p21Cip1 was not colocalized in these cells (Fig. 6D) but did localize in the nuclear compartment of lactotroph and nonlactotroph cells (Fig. 6F). p21Cip1 staining was decreased at day of delivery, with less nuclear and more cytoplasmic expression (Fig. 6E).

Cyclin D1, important for G1 progression, was also differentially expressed with highest expression observed in mid-late pregnancy (Fig. 5, D and E). Levels of cyclin E and cyclins B1 and B2, important for G1/S and G2/M progression, respectively, were stable across the reproductive stages (Fig. 5D).

**Lactational, postweaning, and postpartum pituitary cell cycle response**

As shown above (Fig. 3B), pituitary PCNA protein expression reversed to pregestational levels at 3 wk lactation and 3 wk after weaning. Moreover, elevated PCNA expression observed at the day of delivery also decreased 3 wk postpartum (Fig. 2C), i.e. 3 wk after pup removal at day of delivery. Correspondingly, specific pituitary staining for Ki-67 was undetectable at 3 wk postpartum (Fig. 3N). PCNA expression decreased at all these stages compared with expression observed in nonpregnant mice (Fig. 3B). Generally, expression of pituitary cell cycle regulators reversed to nonpregnant levels at day of delivery or at 3 wk lactation (Fig. 5B). Moreover, p15INK4b and p16INK4a expression revealed a dual pattern after 2 wk pregnancy. After p15INK4b levels decreased to a trough at 3 wk lactation, they increased at 3 wk after weaning. Conversely, p16INK4a peaked at day of delivery and then decreased again at 3 wk lactation (Fig. 5B). Cyclin D1 expression decreased after day of delivery to a lower level than nonpregnancy (Fig. 5D), similar to the observed rebound decrease pattern of PCNA.

The general reversibility of CDK inhibitor levels after delivery was concordant with their expression at 3 wk postpartum, i.e. without a 3-wk lactation period (Fig. 2C). Furthermore, p16INK4a expression was not reversed but rather elevated at 3 wk postpartum, in contrast to the pattern observed at lactation (Fig. 5B).

**Pituitary cell cycle regulation during E2 administration**

Unlike short-term serum E2 level peaks observed during estrus in female mice, longer sustained increased E2 levels are present during the second half of pregnancy (14). We therefore studied long-term E2 administration as a model for lactotroph hyperplasia. As shown above, pituitary cell proliferation increased with E2 administration and at reproductive stages 2 wk pregnancy and day of delivery. Some pituitary cell cycle regulators differentially ex-

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**FIG. 2.** Pituitary gestational expansion, proliferation, and cell cycle alterations reverse partially or completely within 3 wk of pup removal on DOD. A and B, Pituitary weights (milligrams) and pituitary to body weight ratios (milligrams per gram) in female mice at the indicated reproductive stages: non-P, DOD, and 3w-PP (n = 4–12/group). Data are expressed as means ± SD. C, Protein expression of PCNA and the indicated CDK inhibitors that are altered at DOD reverse partially or completely at 3w-PP back to the expression level of the nonpregnant stage. Western blot analysis of the indicated proteins in total protein extracts (30 μg each) from pituitaries of four wild-type mice per time point. **, P < 0.01; †, P < 0.001; ‡, P < 0.0001. Non-P, Nonpregnant; DOD, day of delivery; 3w-PP, 3 wk postpartum and after pup removal on day of delivery.
pressed during E2 administration were comparably expressed at reproductive stage 2-wk pregnancy, including p15INK4b, p16INK4a, p21Cip1, and p27KIP1 (Fig. 5, A, B, and F). p57KIP2 expression levels were not changed with either E2 administration or at reproductive stage 2 wk pregnancy. Concordant with Western blotting results, quantitative PCR detected comparable p15INK4b and p21Cip1 mRNA elevations both during E2 administration and at 2 wk pregnancy (1.9- to 2.3-fold and 4.6- to 5.9-fold, respectively, Fig. 6, A and B). p53 expression was unchanged during pregnancy or E2 administration (data not shown). Whereas cyclins B1 and B2 increased with E2 administration, levels of cyclin D1 did not vary (Fig. 5, C and F). In contrast, cyclin D1 increased at 2 wk pregnancy, but cyclin B1 and B2 levels were not altered at different reproductive stages (Fig. 5D).

In summary, pituitary cell cycle regulation during E2 administration partially recapitulated findings observed during pregnancy, specifically at the reproductive stage of 2 wk of pregnancy.

**Discussion**

Whereas monoclonal lactotroph proliferation underlies prolactinoma formation, the prototype of extensive polyclonal lactotroph proliferation, culminating in pituitary hyperplasia, occurs during pregnancy (4, 5, 15, 16). Ges-
tational lactotroph proliferation may be mediated by hormonal stimuli (4), including E₂ (8, 9). However, E₂- and pregnancy-associated pituitary hyperplasia confers no increased risk of pituitary adenoma (17). In addition, most prolactin tumors in women who achieved pregnancy do not enlarge or become clinically significant (18, 19). There is scant knowledge of molecular regulators stimulating and reversing pregnancy-induced lactotroph replication. In this study we showed that as pituitary weight increases during pregnancy or E₂ administration, the hypophyseal cell cycle response also includes activating antiproliferative mechanisms. Specifically, pituitary p15 INK4b and p21Cip1 are up-regulated during mid-late gestation, coincident with induced proliferation. E₂ administration partially recapitulates gestation-induced pituitary cell cycle expression profiles.

Pituitary expansion at mid-late pregnancy strongly correlates with increased PCNA and Ki-67 expression, and immunostaining of these two proliferation markers demonstrates increased replication from the nonpregnant stage to day of delivery. Previous analyses of pituitary proliferation during rodent and human pregnancy were based mainly on morphological criteria (1–3) including DNA synthesis and PCNA and 5-bromo-2’-deoxyuridine labeling. Proliferative labeling indices showed low lactotroph proliferative activity during pregnancy and lactation and high activity at estrus and the day of parturition. Suckling stimuli in pup-removed lactating rats elicited a marked increase in 5-bromo-2’-deoxyuridine labeling indices in the presence of E₂ (3). Accordingly, we found that lactotroph proliferation also peaked at day of delivery. However, the kinetic analysis reveals that this proliferation starts as early as the end of second week of pregnancy. Indeed, several studies have reported increased lactotroph mitosis in women at earlier stages of human pregnancy (4, 5) in concordance with our murine results.

In addition to gestational pituitary expansion, our results show elevated pituitary proliferation markers during E₂ administration. Pituitary hyperplasia was confirmed by the presence of intact reticulin fibers during either mid-late pregnancy or E₂ administration. As predicted, parallel staining for prolactin and either PCNA or Ki-67 indicated that lactotroph cells comprise the main proliferating pituitary cell type during E₂ administration, pregnancy (2 wk pregnancy), and immediate peripartum stage (day of delivery).

The present results also confirm reversal of proliferative pituitary activity postpartum or after the late lactative phase, possibly related to low postpartum serum E₂ levels (14, 20). Surprisingly, decreased molecular proliferation marker expression, including PCNA and Ki-67, after the day of delivery did not translate to pituitary mass reorganization. The striking increase in pituitary weight during pregnancy does not reverse but rather stabilizes throughout lactation and postweaning stages. Nonproliferative processes including cytoplasmic accumulation may comprise the main component of gestational pituitary weight gain, whereas pituitary cell proliferation may be a minor contributor. In support of this, Castrique et al. (21) reported that in prolactin-Cre/ROSA-YFP transgenic mice,
cell volumes of prolactin-positive pituitary cells increased significantly by midlactation.

Alterations in cell cycle regulators such as p27Kip1, p18 INK4c, and high mobility group protein A-2 can lead to pituitary neoplasia or hyperplasia development (10, 22–24). CDK4 is critical for regulation of lactotroph mass and function, especially postnatal cell proliferation (12, 13). We now demonstrate the span of cell cycle regulators in the mouse pituitary gland during E2 administration and during pregnancy, lactation, and the postweaning period. In E2-administered mice, regulators were shown to be differentially expressed, including INK4 inhibitors, CIP/KIP inhibitors, and cyclins. Generally, the protein expression profile of cell cycle proteins, including p15INK4b, p16 INK4a, p21 Cip1, and p27Kip1, in the expanding pituitary during E2 administration partially mimics effects of pregnancy on pituitary cell cycle, specifically at 2 wk pregnancy. Pituitary CDK4 activity during pregnancy may be positively regulated by association with cyclin D1 and by decreased p16 INK4a. Noteworthy, expression of these proteins is frequently altered in human pituitary tumors (13, 25–28). Furthermore, up- or down-regulation of pituitary cell cycle regulators detected at mid-late pregnancy are reversed to pregestational levels postpartum. Serum E2 levels during murine gestation are low in early pregnancy, increase during mid-late pregnancy up to d 17 and then decrease rapidly after delivery and throughout lactation (14, 20). Thus, expression patterns of pituitary cell cycle regulators during the last week of pregnancy suggests a mechanistic role for E2.

Both p15INK4b and p21Cip1 act as negative regulators of CDK4 and CDK2, respectively, impacting cell proliferation in vitro and in vivo. p21Cip1 also blocks S phase progression by inhibiting PCNA activity, thus causing cell cycle arrest (29). p15INK4b induction was associated with decreased proliferative activity in rat lactosomatotroph GH3 cells treated with TGF-β1 (30). Elevated p15INK4b and p21Cip1 expression in pituitary hyperplastic states in vivo may be important for the cell cycle response to E2 administration and to pregnancy. The role for p21Cip1 in regulating pituitary mass in the adult mouse and human GH- and prolactin-secreting pituitary adenomas (31, 32) implies that p21Cip1 may regulate pituitary trophic ho-

FIG. 5. Protein expression of CDK inhibitors and cell cycle regulators is differentially expressed in female mice administered placebo or E2 for 2.5 months (A, C, and F) and in females at the indicated reproductive stages (B, D, and E). Protein expression profile during E2 administration partially mimics reproductive stage P2w. Western blot analysis of the indicated proteins in total protein extracts (30 μg each), each isolated from four pituitary glands per time point. Levels of the indicated proteins are determined by densitometry (E and F). Values are expressed as means ± se. *, P < 0.05; **, P < 0.01. Non-P, nonpregnant; P2w, 2-wk pregnancy; DOD, day of delivery; L3w, 3 wk lactation; 3w-post-W, 3 wk after weaning; cyc D1, cyclin D1.

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meostasis by constraining tumorigenesis when exposed to pro-proliferative stimuli.

E2 activates p21Cip1 expression through direct promoter regulation in MCF-7 breast cancer cells (33). We show elevated pituitary p21Cip1 expression during E2 administration and gestation, without elevated p53 expression. Whereas cell growth-inhibiting activity of p21Cip1 correlates with nuclear localization, p21Cip1 relocalization to the cytoplasm promotes cell survival and proliferation (29). Estrogen stimulates p21Cip1 and uterine cell proliferation, whereas cotreatment with progesterone prevents both effects (34). In our study, intranuclear p21Cip1 localization was evident mainly at 2 wk pregnancy. Furthermore, Ki-67, a marker of cycling cells, did not coexpress with nuclear p21Cip1, suggesting p21Cip1-growth-inhibiting activity. Progesterone levels increase throughout pregnancy (14, 20) and are not associated with lactotroph replication. Thus, the gestational estrogen milieu may potentiate pituitary proliferation and p21Cip1 transcriptional induction at 2 wk pregnancy. Concomitantly, other gestational-related factors mediate p21Cip1 nuclear location restraining pituitary proliferation. At the day of delivery, with the altered hormonal milieu, and effects of unopposed serum E2, p21Cip1 may be transported to the cytoplasm to mediate suckling and E2-induced pituitary proliferation. This may explain higher pituitary proliferation rates at the day of delivery compared with 2 wk pregnancy. During E2 administration, we demonstrated increased pituitary proliferation and nonnuclear p21Cip1 expression, suggesting that the E2-induced p21Cip1 indeed promotes rather than inhibits replication. Simultaneously, the p16INK4a levels increase at day of delivery, potentially attenuating the pituitary proliferative drive at this stage.
This study has several limitations. First, we recognize that pituitary cell cycle control may reflect species and strain specificity. Second, the lack of adequate functional differentiated mouse or human lactotroph cell cultures makes comprehensive cell cycle analysis difficult, and much insight into human pituitary cell proliferation is derived from *in vivo* animal studies. Third, cell cycle regulators are likely only some of several target genes required for pituitary hyperplasia and partial postpartum involution. Unique growth factor regulation of lactotroph cell growth and function also likely contributes to pituitary modeling (35). Furthermore, additional pituitary, ovarian, fetal, and placental interrelationships are also likely operative during gestation (36).

Comparison of two lactotroph hyperplasia paradigms, E2-induced and gestational/suckling-induced, suggests diverse mechanisms used by the pituitary gland to expand mass. Some genes investigated are not concordantly induced in the two models, suggesting that pituitary mass modulation is activated by diverse signaling mechanisms, depending on physiological and pharmacological conditions. In support of this notion, several cell cycle components likely control pituitary mass homeostasis through both pro-proliferative and inhibitory signals. Gestational, lactational, and postweaning pituitary cell cycle progression and arrest likely follow an orchestrated temporal cascade determined by the hormonal milieu including E2 levels.

We propose a model for this cascade in gestational and E2-induced pituitary hyperplasia (Fig. 7). Pregnancy and E2 stimuli are required to induce pituitary proliferation through the down-regulation of p16INK4a and p27Kip1. Increased p15INK4b expression may attenuate pituitary expansion and delay its kinetics. E2 may also trigger the p21Cip1 promoter, but p21Cip1 is not localized to the nucleus during E2 administration, suggesting a role in promoting proliferation. At mid-late pregnancy, *i.e.* 2 wk pregnancy, nuclear p21Cip1 localization regulated by

![Proposed model for pituitary cell cycle regulation during mid-late pregnancy (P2w) and during E2 administration in WT female mice.](https://academic.oup.com/endo/article-abstract/153/10/5011/2424521)

Pregnancy and E2 administration induce alterations in levels of pituitary cell cycle regulators in a G1/S-dependent manner. The altered levels can either stimulate or inhibit pituitary cell cycle progression and proliferation. Some alterations induced by E2 administration are mimicked by pregnancy, and others are unique for either one of these stimuli. p21Cip1 exhibits a dual role. E2 administration also induces cyclins B1 and B2, which have a role in cell cycle progression distal to the G1/S point. Macroscopically, the pituitary is remodeled from a normal to a hyperplastic gland, without formation of pituitary tumors. Cell cycle alterations are reversible after pregnancy, at postpartum, and postlactational stages. P2w, 2-wk pregnancy.
nonestrogenic factors may limit pituitary proliferation. p21\textsuperscript{Cip1} possibly represents a rate-limiting pathway, preventing a more robust proliferative response to pregnancy. We show that p15\textsuperscript{INK4b} and p21\textsuperscript{Cip1} levels closely mirror proliferative profiles throughout pregnancy, further suggesting that these components of cell cycle control determine pituitary homeostasis, also in the setting of pharmacological E\textsubscript{2} administration. We hypothesize that pituitary p21\textsuperscript{Cip1} up-regulation has unique functions, either preventing or promoting excessive pituitary cell replication. Thus, p21\textsuperscript{Cip1} may be a promising target for investigating therapeutic manipulations of pituitary neoplasms, specifically of lactotroph and/or somatotroph origin.

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